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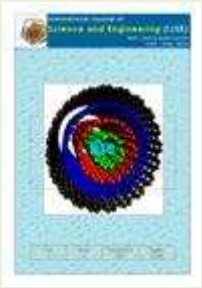

Immobilized bacteria by using PVA (Polyvinyl alcohol) crosslinked with Sodium sulfate

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Abstract - A new bacteria immobilization technique using PVA (polyvinyl alcohol) crosslink with sodium sulfate was developed. This new technique can simultaneously eliminate the agglomeration of PVA beads and the toxicity of boric acid caused by the PVA-boric methods, also reducing the swelling (when soaking in water) of PVA-boric methods. Beads were immobilized by using four different PVA entrapment processes to create group B, group N, group P and group S. The stability, swelling, relative mechanical strength of the PVA beads were compared in this study. Only group S was the best and chosen to do experiment for checking survival of bacteria after immobilization process and TOC removal performance of anaerobic reactor. The TOC removal performance of anaerobic reactor achieved 80-87%.

Key words: *PVA-alginate beads; immobilized bacteria; bacterial gel bead.*

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1. Introduction

The enzymes and bacteria are the core of the biological treatment process from the activated sludge, anaerobic treatment, to the anammox. All these processes are considered to be economical effective treatment if possible to reduce the hydraulic retention time in these process; increase the processing load of organic matter, it means that the treatment reactor will be smaller and reduce energy consumption for mixing and pumping. All most of the current biological process then bacteria exist in suspended sludge form, there are some following disadvantages: 1- Difficult to control the concentration of sludge in treatment reactors; 2- Waste-out of sludge from reactors. 3- The suspended sludge is very difficult settling in mechanical treatment process. Thus, required the design of the treatment system with long hydraulic retention time (HRT) and/or reactor with big volume. So, if we want to decrease the HRT and easily to control the sludge (Bacteria) concentration, we need to have solutions to immobilize bacteria.

Immobilization of living cells has become a technique for increasing the productivity of biochemical engineering processes. One of the most widely used techniques for bacterial immobilization is bacterial entrapment, in which, the bacteria (living cells) are enclosed in a polymeric matrix which has porous enough to allow the diffusion of substrates to the bacteria and of products away from the bacteria. Materials which have been successfully used for cell entrapment include agar, agarose, kappa-carrageenan, collagen, alginate, chitosan, polyacrylamide, polyurethane, and cellulose [1]. However, each of these polymers has drawbacks, such as poor mechanical strength and durability (agar, agarose, kappa-carrageenan, collagen, alginates, chitosan), toxicity to microorganisms (polyacrylamide, polyurethane), or high cost [2, 3].

The use of poly vinyl alcohol (PVA) as an immobilization matrix was initiated about more than 30 years ago by Freeman and Aharonowitz [4]. A few years later, PVA gels have been successfully used in bioremediation particularly in wastewater treatment [2, 5]. Besides that, PVA is the largest synthetic water soluble polymer produced in the world [6]; and PVA offers various advantages over the conventional alginate hydrogels including lower cost, higher durability and chemical stability, and its non-toxicity to viable cells [7]. PVA gel exhibits a high degree of swelling in water (or biological fluids) and it is also rubbery and elastic in nature [8]. The highly hydrophilic PVA must be cross-linked either chemically or physically to make it insoluble [9]. Ariga et al [2] used the technique of iterative freezing and thawing of PVA to form a gel suitable for cell immobilization. They found that this technique produced a low-cost material with a rubber-like elasticity and high strength. Hashimoto and Furukawa [5] used a simpler and less energy-intensive method for PVA immobilization.

Crosslink the PVA used a boric acid solution. The PVA-boric acid technique provides an easy method of immobilization cell, producing elastic beads of high strength and durability [5]. There are two potential problems with this technique, however. The saturated boric acid solution used to crosslink the PVA is highly acidic (pH of approximately 4), thus could cause difficulty in maintaining cell viability. In addition, PVA is an extremely sticky material. PVA beads, therefore have a tendency to agglomerate [10]. This is particularly a problem in applying PVA-immobilized cells to fluidized bed reactors.

In this study, the agglomeration and swelling problem of the PVA-boric acid method are eliminated by the addition of a small amount of calcium alginate. After that, other different kinds of bead are performed by soaking PVA-boric in solutions of Na_2SO_4 - 0.5 M; $NaNO_3$ - 0.5 M and NaH_2PO_4 - 0.5 M, respectively. The main focus of this study is to investigate the feasibility of these methods; to find the reasonable ratio between PVA, sodium alginate and sludge used in forming beads; to compare relative strength between kinds of produced beads; to check survival of bacteria after immobilization process; to test effective of anaerobic reactor when presence of beads.

2. Materials and methods

2.1. Activated sludge

Sludge was collected from the Kim Lien Wastewater Treatment Plant in Hanoi, Vietnam.

2.2. Chemicals

Polyvinyl Alcohol - PVA $(C_2H_4O)_n$ was bought from Kuraray Co.,ltd (Singapore). Grade is 98%-100% hydrolyzed and molecular weight 77000 [11].

Sodium Alginate $(C_6H_7O_6Na)n$; Calcium Chloride ($CaCl_2$); Boric acid (H_3BO_3); Sodium Sulfate (Na_2SO_4); Sodium Nitrate ($NaNO_3$); MonoSodium Phosphate (NaH_2PO_4); $C_6H_{12}O_6$; Wilson Blair agar were purchased in Hanoi, Vietnam.

2.3. Methods of immobilization

Prepare these following solutions: 500 ml of 0.5 M NaH_2PO_4solution; 500 ml of 0.5 M Na_2SO_4solution; 500 ml solution with saturated H_3BO_3 (5-7% w/v) and added $CaCl_2$ (2% w/v); 500 ml of $NaNO_3$ solution (50% w/v); 50ml of Sodium alginate solution (5% w/v).

Add water to a beaker (was available 10g of PVA) to obtain 70ml solution. This solution was then heat to a temperature of around 80°C to dissolve PVA. 20 ml Sodium alginate solution (5% w/v) was added to the PVA solution. The PVA-alginate solution was then cooled to around room temperature. Next, 10 ml of activated sludge was added in the PVA-alginate solution. In fact, this solution has PVA (10%, w/v), Sodium alginate (1%, w/v), activated sludge (10%, v/v) [11]. We changed concentration of PVA and sodium alginate to have some different solutions for comparing. This solution was named A solution.

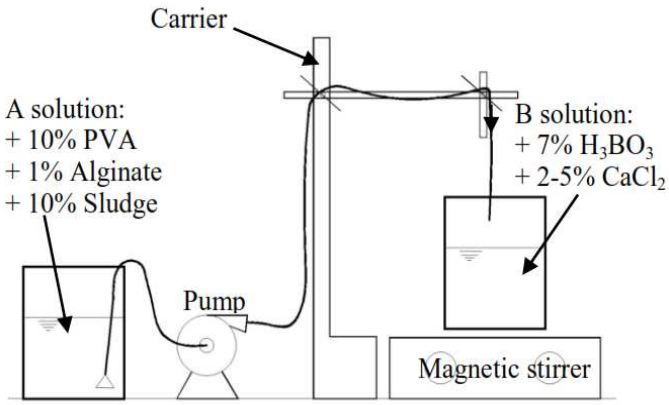

Fig. 1: *Diagram of creation PVA beads*

The A solution was dropped into B solution: saturated H_3BO_3 (7%, w/v) and $CaCl_2$ (2%, w/v). And immersed for 1 hour to form PVA-Boric beads (group B) (**Fig. 1**). The beads were then removed, washed and stored at 4 °C in the pure water. PVA-phosphate beads (group P) were prepared by transferring the beads (group B) to 0.5M NaH_2PO_4 solution and immersing for 1 hour. PVA-sulfate beads (group S) were prepared by transferring the beads (group B) to 0.5M Na_2SO_4 solution and immersing for 1 hour. PVA-nitrate beads (group N) were prepared by transferring the beads (group B) to 50% $NaNO_3$ solution and immersing for 1 hour. (All beads were then removed, washed and stored at 4°C in the pure water until using).

2.4. Test of relative mechanical strength

The Rotana 460 centrifuge (HitechCo.,ltd) with 4-plates in Swing-out rotor was used to determine the relative mechanical strength of the PVA beads. Radius of rotary was 203 mm. The beaker was 62 mm in diameter, 137 mm in height. Twenty beads of various kinds were added to the beaker and the water level adjusted with pure water to 10 cm in height. The centrifuge was controlled from 500-3000 rpm. The beads were agitated in the beaker for 5 min and the surviving beads counted.

2.5. Test swelling of beads in the water

The beads will use for wastewater treatment. Thus, experiment to determining mechanical strength of beads in the water is necessary. 20 beads of various kinds were dropped into the water bottle (size 500 ml), and the beads were stirred by magnetic stirrer machine. Daily, These bottles water were changed pure water inside them and observation was memorized.

2.6. Check survival of bacteria after immobilization

The sludge (which was composition of the beads) had many spores of sulfite-reducing bacteria. If these spores can still survival after immobilization process, it means this immobilization method is suitable. To check survival of bacteria, this study used Wilson Blair agar as an indicator.

Some beads of the best kind in bead types were washed by 0.9% NaCl solution. Wilson Blair agar in a glass tube was taken from refrigerator, and heated at 90°C about 30 min to be hydrolyzed completely. After

that, glass tube was decreased temperature to 45°C. Finally, some beads were inserted inside Wilson Blair agar. This glass tube was kept at 37°C and observed following 24h, 48h.

2.7. Test of performance in anaerobic reactor

The best kind of bead types was released into a reactor to check effective treatment of reactor when appearing the beads. **Fig. 2** shows schematic diagram of the reactor system. The reactor had a volume of 500ml; Volume of beads was 50 ml.

Temperature of the reactor was kept by water tank at 37°C [12]. Substrate and nutrients water in this study follow as Bach, Bhatti [13]. The synthetic wastewater consisting components as shown in **Table 1**; and **Table 2** was used as trace substrate. Sucrose ($C_6H_{12}O_6$) was used as the sole carbon source in feeding, concentration of TOC in water was kept at 5g/l. N and P were added in the form of NH_4Cl and KH_2PO_4 in accordance with COD:N:P ratio = 300:5:1[14].

At fixed time in everyday, aspirate 51 ml of water from the reactor. After that, inject 50 ml of nutrient water and 1 ml of saturated sodium bicarbonate as a buffer.

TOC and pH of effluent water were measured follow as standards in **Table 3**.

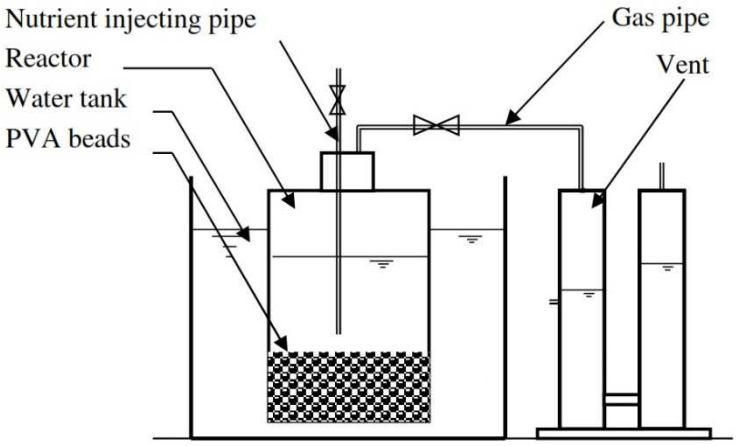

Fig. 2: *Schematic view of the reactor system*

Table 1: *Compositions of synthetic wastewater*

Components	Concentration
$C_6H_{12}O_6$	12.5 g/l (5g-C/l)
NH_4Cl	According to the ratio of C:N:P
KH_2PO_4	According to the ratio of C:N:P
$MgSO_4.7H_2O$	0.1 g/l
Trace nutrient solution	10 ml/l

Table 2: *Concentration of trace nutrient solution*

Trace nutrient	Concentration (mg/l)
$FeCl_2.6H_2O$	4.9
$MnCl_2.4H_2O$	0.35
$CoCl_2.6\ H_2O$	0.085
$ZnCl_2$	0.35
$NiCl_2.6\ H_2O$	0.42
$CaCl_2.2\ H_2O$	0.35
H_3BO_3	0.035
$Na_2MoO_3.2\ H_2O$	0.085
$CuCl_2.2\ H_2O$	0.009

Table 3: *Analytical parameters and analytical methods*

Parameters	Analytical frequency	Analytical methods	Instruments
pH	One time/ day	ISO 10523:2008	pH 720 inolab (WTW- Germany)
TOC	One time/ day	ISO 8245: 1999	TOC-Vcph (Shimadzu-Japan)

3. Results and discussion

3.1. Study of immobilization method

Two potential problems in PVA-boric method are the agglomeration of PVA gel beads and the toxicity of saturated boric acid [15]. Wu and Wisecarver [10] added a small amount of calcium alginate to prevent the agglomeration of PVA gel beads. Chen and Lin [16] reduced the immersion time of saturated boric acid from 15–24 hours to 10 min–2 hours to diminish cell damage and used an orthophosphate solution for gel strength reinforcement. The first was formation of calcium alginate membrane in **Fig. 3**. After that, these borate ions will crosslink the alcohol groups on adjacent chains as depicted in **Fig. 4**.Structure of beads bases on both links PVA-boric and Calcium-Alginate.

However, due to the aforementioned unusual semi solid properties, the beads produced could easily dissolve in distilled water. To overcome this problem, ion sulfate, ion nitrate and ion phosphate were respectively introduced, as these ions possess the ability to form linkages among the cross-linked PVA. Bead of PVA-nitrate was presented by Chang et al. [15]; Bead of PVA-Phosphate was presented by Chen et al. [16]. Pillay et al.

[17] have also introduced sodium sulfate as their crosslinking agent together with boric acid to crosslink the PVA. The overall reaction that took place during PVA–alginate bead formation is shown in **Fig. 5.** Similar that, Beads of group N and group P were created.

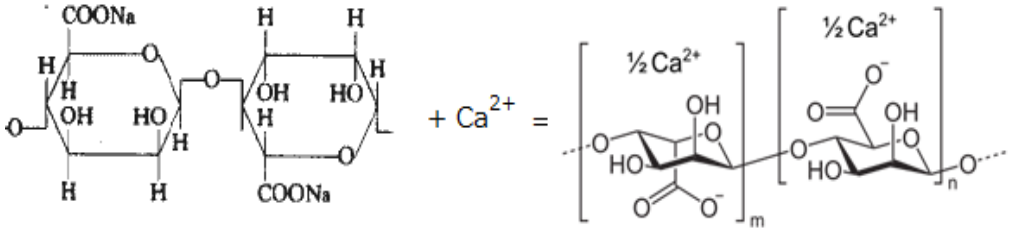

Fig. 3*: Formation of Calcium alginate membrane[18]*

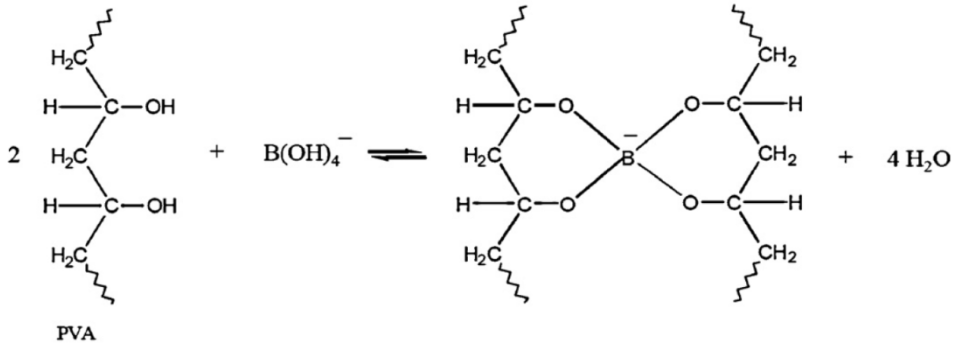

Fig. 4*: Formation of PVA-Boric[19]*

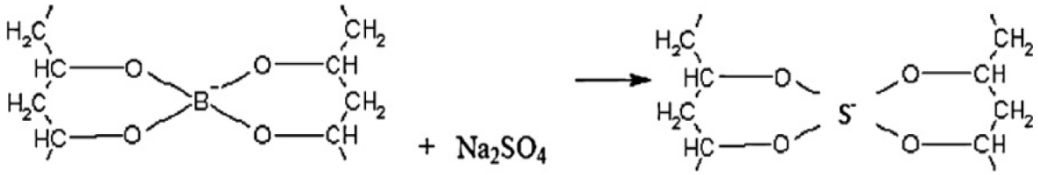

Fig. 5*: Formation of PVA- sulfate [11]*

3.2. Effect of PVA and Alginate ratios on formation of group B beads

The beads of group B were prepared according to the method explained in Section 2.3. PVA contributed strength and durability to the bead, whereas calcium alginate improved their surface properties, reducing the tendency to agglomerate. The percentage of PVA in the beads was kept in the range of 10-12% (w/v) as recommended for maximum bead strength. Various percentage of PVA was attempted for the immobilization procedure (**Table 4**). The percentage of alginate in the beads was also varied for immobilization to find the best percent (**Table 5**).

Table 4 - *Influence of the percent of PVA to formation of group B beads*

PVA (%, w/v)	Agglomeration	Formation of beads	Strength
6	prevented	Not good	No formation
8	prevented	Oval shape	weak
10	prevented	good	Strong
12	prevented	good	Strong
14	prevented	have tail	Strong

Table 5 - *Influence of the percentage of alginate to formation of group B beads. (Percentage of PVA was kept at 10%)*

Agglomeration	Formation of beads	Strength
Not prevented	Not formation	No formation
prevented	Oval shape	weak
prevented	Oval shape	good
prevented	good	good
prevented	good	good

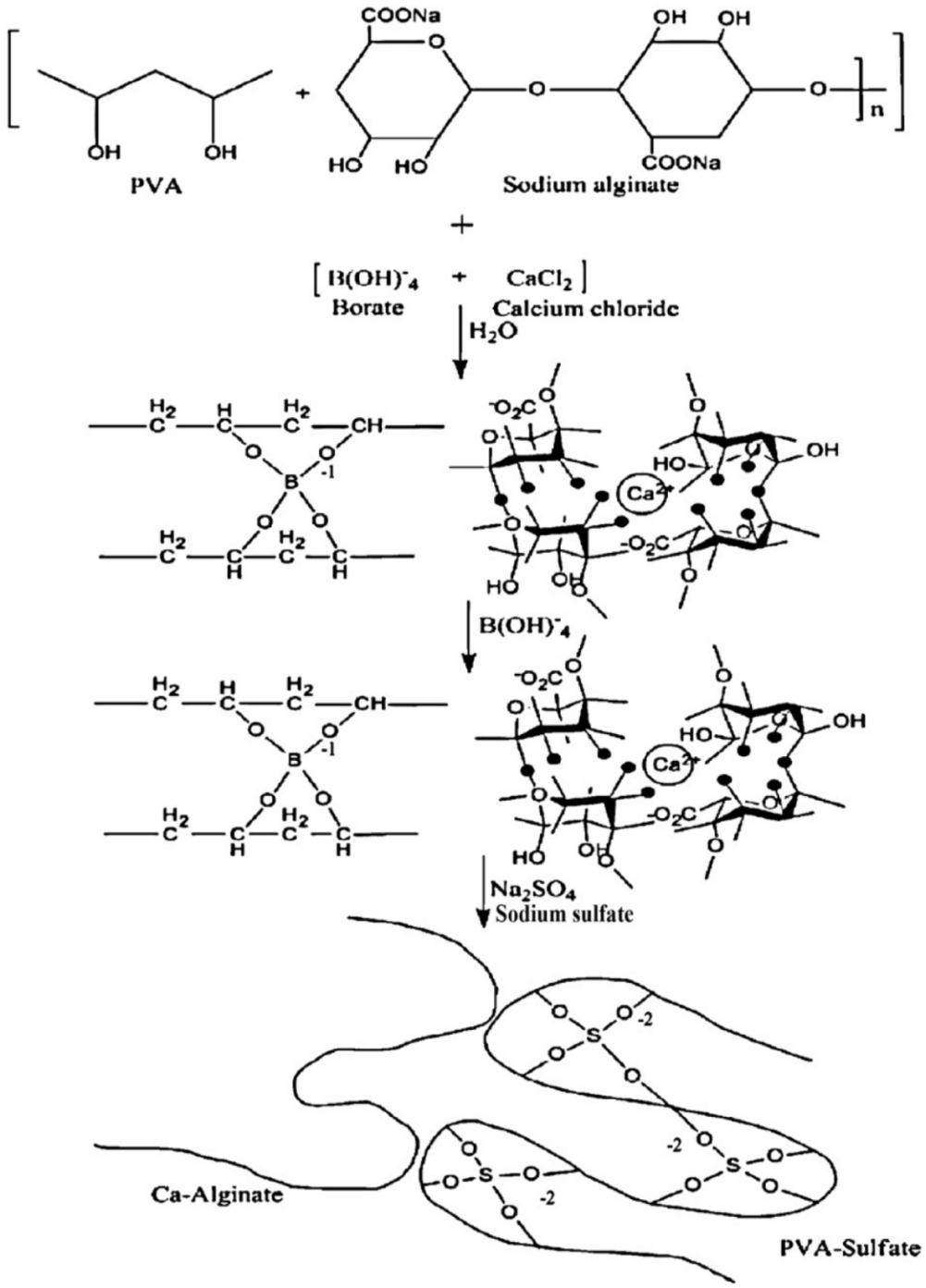

Fig. 6*: Formation of beads (group S) [11]*

3.3. Test of relative mechanical strength

To could assess mechanical strength, beads were centrifuged. The relative mechanical strengths of all kinds of bead are shown in **Fig. 7**. The group N had a very strong mechanical strength and are very difficult to break; they were not broken at agitation speeds lower than 2500 rpm. The group P showed a relatively weak mechanical strength, which might have been due to insufficient cross-linked, and were all broken when agitated at speeds over 2000 rpm. The group S had a medium mechanical strength.

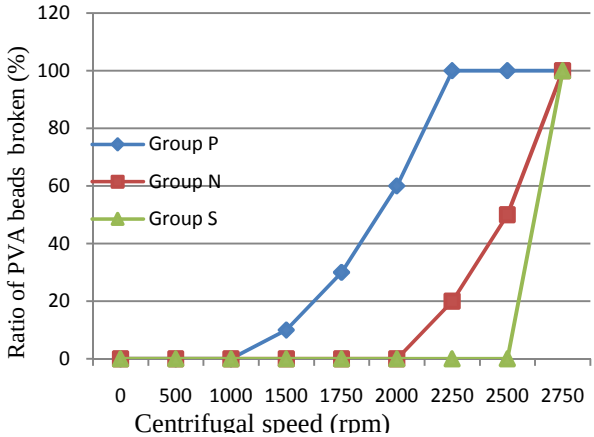

Fig. 7: *Relative mechanical strengths*

3.4. Test swelling of beads in the water

The swelling of beads is shown in **Table 6** and **Fig. 8**. The Group B was the weakest. The Group S was the strongest, they were almost be swelling very little, and special didn't solute in water. Although, the group N and Group P didn't solute, but they were be swelling much, from 4 mm in diameter at first to 7 mm and 8 mm in diameter at last, respectively. Wu and Wisecarver[10]suggested that the agglomeration problem is due to the insufficient crosslinking of the PVA by boric acid. The PVA beads of group B which were not sufficiently crosslinked, could be dissolved almost completely after transferring into pure water for a few hours. After a few hours, the beads of group N and group P were exhibited a very weak gel structure[17].

Fig. 8:*Beads swollen after immersing in water*

Table 6: *The swelling of beads with temporal trend*

Time	Group B	Group N	Group P	Group S
0	4.0 mm	4.0 mm	4.0 mm	4,0 mm
2 h	4.0 mm	4.0 mm	4.5 mm	4,0 mm
1 day	4.5mm	4.5 mm	5.0 mm	4,5 mm
2 days	4.5 mm	5.5 mm	6.0 mm	4,5 mm
3 days	5.0 mm	6.0 mm	7.0 mm	4,5 mm
4 days	5.5 mm	6.5 mm	7.0 mm	4,5 mm
5 days	5.5 mm	6.5 mm	8.0 mm	4,5 mm
7 days	5.5 mm	6.5 mm	8.0 mm	4,5 mm
14 days	5.5 mm	6.5 mm	8.0 mm	4,5 mm

3.5. Check survival of bacteria after immobilization

In Wilson Blair agar was available sodium sulfate and iron (III) chloride. In anaerobic condition, sulfite-reducing bacteria digests sulfate to hydrosulfide (H_2S). Hydrosulfide reacts with iron (III) to create FeS - black color. So, if Wilson Blair agar changes color after transplant beads inside, it means bacteria in beads can exist after immobilization process.

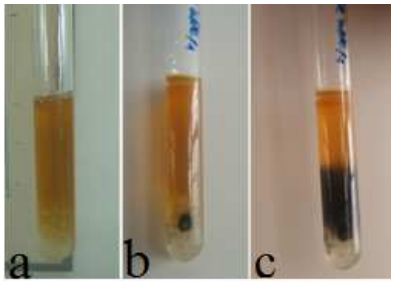

Fig. 9: *Beads of group S were transplanted in Wilson Blair agar: a) after 2 hours; b) after 24 hours; c) after 72 hours.*

In this study, several beads of group S were transplanted inside Wilson Blair agar. Result (**Fig. 9**) demonstrated that this immobilization process is suitable.

3.6. Test of performance in anaerobic reactor

TOC removal performance of reactor (with presence of group S inside) is shown in **Fig. 10**. In first five days, effective treatment is only under 30%, TOC residue is more and more arise after each add nutrient, pH in reactor is in low level (<3.5). From fifth day and eighth day, pH is adjusted respectively by adding more 2 ml and 3 ml of saturated $NaHCO_3$ solution; pH oscillates around 6.2 to 6.5. From sixteenth day, performance of reactor increases and is stable at 80-87%.

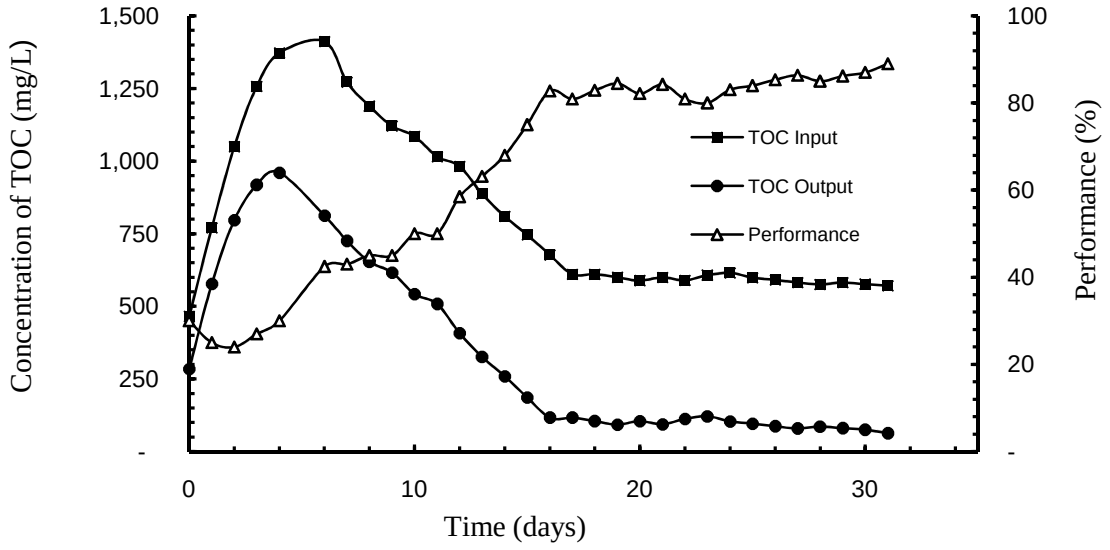

Fig. 10: *TOC removal performance*

4. Conclusion

PVA is a cheap, non-toxic material and suitable for cell immobilization. But the agglomeration of PVA beads and the dissolve back of group B are two problems that are encountered when using the PVA-boric acid method. Adding a small amount of sodium alginate in the PVA gel can prevent the agglomeration of PVA beads [10]. Group N, group B and group P overcame both above disadvantages, but they swollen in water. Thus, it's not suitable for this research purpose. Only group S was the most suitable with the immersion time for solidification is very short; well-non swollen in water; the high mechanical strength. Especially, the TOC removal performance in the anaerobic reactor increased clearly with presence beads (group S). The PVA-sulfate method may be a promising and economical technique for cell immobilization.

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ISO 10532:2008 – Water quality – Determination of pH

ISO 8245: 1999 - Water quality – Guidelines for the determination of total organic carbon (TOC) and dissolved organic carbon (DOC).